HIGH VISCOSITY XANTHAN POLYMER PREPARATIONS

[01] This application claims the benefit of provisional application U.S. Serial No. 60/456,245 filed March 21, 2003.

FIELD OF THE INVENTION

[03] The invention relates to the field of microbial products. In particular it relates to microbial products having improved properties for various industrial purposes.

BACKGROUND OF THE INVENTION

[04] The chemical structure of xanthan is composed of a linear cellulosic (1→4)-β-D-glucose polymer with trisaccharide side chains composed of mannose, glucuronic acid and mannose, attached to alternate glucose residue in the backbone. (Milas and Rinaudo, Carbohydrate Research, 76, 189-196, 1979). Thus xanthan can be described as a branched chain polymer with a pentasaccharide repeat unit; normal xanthan typically has 2000-3000 pentasaccharide repeat units. The xanthan polymer is typically modified by acetylation and pyruvylation of the mannose residues.

[05] The fermentation of carbohydrates to produce the biosynthetic water-soluble polysaccharide xanthan gumBy the action of *Xanthomonas* bacteria is well known. The earliest work was conducted by the United States Department of Agriculture and is described in U.S. Pat. No. 3,000,790. *Xanthomonas* hydrophilic colloid ("xanthan") is an exocellular heteropolysaccharide.

[06] Xanthan is produced by aerobic submerged fermentation of a bacterium of the genus *Xanthomonas*. The fermentation medium typically contains carbohydrate (such as sugar), trace elements and other nutrients. Once fermentation is complete, the resulting fermentation broth (solution) is typically heat-treated. It is well established that heat treatment of xanthan fermentation broths and solutions leads to a conformational change of native xanthan at or above a transition temperature (T_M) to produce a higher viscosity xanthan. Heat treatment also has the beneficial effect of destroying viable microorganisms and undesired enzyme activities in the xanthan. Following heat-treatment, the xanthan is recovered by alcohol precipitation. However, heat treatment of xanthan fermentation broths also has disadvantages, such as thermal degradation of the xanthan. Heating xanthan solutions or broths beyond T_M or holding them at temperatures above T_M for more than a few seconds leads to thermal degradation of the xanthan. Degradation of xanthan irreversibly reduces its viscosity. Accordingly, heat treatment is an important technique with which to control the quality and consistency of xanthan.

[07] Xanthan quality is primarily determined by two viscosity tests: the Low Shear Rate Viscosity ("LSRV") in tap water solutions and the Sea Water Viscosity ("SWV") in high salt solutions. Pasteurization of xanthan fermentation broths at temperatures at or above T_M has been found to yield xanthan of a higher viscosity as indicated by higher LSRV and SWV values.

[08] Xanthan polymer is used in many contexts. Xanthan has a wide variety of industrial applications including use in oil well drilling muds, as a viscosity control additive in secondary recovery of petroleum by water flooding, as a thickener in foods, as a stabilizing agent, and as a emulsifying, suspending and sizing agent (Encyclopedia of Polymer Science and Engineering, 2nd Edition, Editors John Wiley & Sons, 901-918, 1989). Xanthan can also be used in cosmetic preparations, pharmaceutical vehicles and similar compositions.

[09] There is a need in the art to produce a xanthan polymer with higher specific viscosity characteristics in the unpasteurized state. Such a higher specific viscosity xanthan polymer could provide more viscosity at equivalent xanthan concentrations, for example, for food, industrial, and oilfield applications.

BRIEF SUMMARY OF THE INVENTION

[10] In a first embodiment an unpasteurized xanthan composition is provided. The composition can be provided by a cell which over-expresses *gumB* and *gumC*. It has an intrinsic viscosity which is at least 20 % greater than xanthan from a corresponding strain which does not over-express *gumB* and *gumC*.

[11] In a second embodiment a xanthan composition is provided. It comprises a population of xanthan molecules having a range of molecular lengths. At least 1 % of the population has a length greater than 3 um as measured by atomic force microscopy.

[12] In a third embodiment of the invention a method is provided for producing a xanthan polymer preparation having increased viscosity relative to that produced by a wild-type strain. The amount of gene product of *gumB* and *gumC* is selectively increased in a *Xanthomonas campestris* culture. The amount of a gene product of *orfX* is not selectively increased. Nor is the amount of a product of a gene selected from the group consisting of *gumD-gumG* selectively increased. A higher viscosity xanthan polymer preparation is thereby produced by the culture.

[13] In a fourth embodiment of the invention a method is provided for producing a xanthan polymer preparation having increased viscosity relative to that produced by a wild-type strain. A *Xanthomonas campestris* strain is cultured in a culture medium under conditions in which it produces a xanthan polymer. The strain selectively produces relative to a wild-type strain more gene product of *gumB* and *gumC* but not of *orfX* nor of a gene selected from the group consisting of *gumD-gumG*.

[14] In a fifth embodiment of the invention an unpasteurized xanthan composition is provided. The composition is made by a cell which over-expresses *gumB*

and *gumC*. The composition has a seawater viscosity which is at least 10 % greater than xanthan from a corresponding strain which does not over-express *gumB* and *gumC*.

[15] The present invention thus provides the art with xanthan compositions which have increased viscosity relative to those similarly produced by corresponding wild-type strains.

BRIEF DESCRIPTION OF THE DRAWINGS

[16] Figure 1 shows genetic constructs relative to a genetic map of the *gumB-M* operon, also known as the *xpsB-M* (xanthan polysaccharide synthesis) operon.

[17] Figures 2A and 2B show Western blot analyses of *gumB* and *gumC* protein product expression, respectively.

[18] Figure 3 shows an intrinsic viscosity plot for xanthan gum samples, one of which over-expresses *gumB* and *gumC* gene products due to the presence of a plasmid carrying extra copies of the genes.

DETAILED DESCRIPTION OF THE INVENTION

[19] It is a discovery of the present inventors that overexpression of *gumB* and *gumC* gene products relative to other genes in their operon, yields xanthan products with higher viscosity on a per weight basis. While applicants do not wish to be bound by any particular theory of operation, it appears that a shift in the ratio of certain gene products leads to a shift in the size distribution of xanthan polymer molecules. A significant number of molecules are of higher molecular length than when xanthan is made by a wild-type cell. These longer molecules lead to a higher viscosity of the population or preparation.

[20] It is known in the art that increases in viscosity can be obtained by pasteurizing xanthan preparations. See Talashek et al., U.S. 6,391,596. However, the increased viscosity found as the result of overexpression of *gumB* and *gumC* is observed even in the absence of pasteurization. Nonetheless subsequent pasteurization of the products of the present invention will yield an even more viscous preparation.

[21] Overexpression of both *gumB* and *gumC* appear to be required to achieve the increased viscosity. When either gene was tested alone, the increase was not observed. The overexpression of *gumB* and *gumC* can be assessed relative to other genes of the *gumB-M* operon. While overexpression relative to any of those genes may be sufficient to achieve the effect, overexpression with respect to *orfX* and *gumD* may be particularly significant. *OrfX* is a small open reading frame that was previously published as a segment of the genome designated as *gumA,* immediately upstream of *gumB.* Recently two open reading frames have been discerned in the former *gumA* region, *ihf* and *orfX.* Overexpression relative to all of the genes *gumD-gumM* may be desirable.

[22] Overexpression of the desired gene products may be achieved by any means known in the art, including, but not limited to, introducing additional copies of the genes encoding the desired gene products to a *Xanthomonas campestris* cell or other bacterium that makes xanthan, and induction of the desired gene products using for example an inducible promoter. Other bacteria that make xanthan include those that have been genetically engineered to contain the xanthan biosynthetic genes. The *gumB* and *gumC* genes can be introduced on one or more vectors, *i.e.,* in combination or individually.

[23] Inducible promoters which can be used according to the invention include any that are known in the art, including the *lac* promoter, the *ara* promoter, the *tet* promoter, and the *tac* promoter. Natural and artificial inducing agents for these promoters are known in the art, and any can be used as is convenient. Additional copies of genes can be introduced on plasmids or viral vectors, for example. Additional copies of the desired genes can be maintained extrachromosomally or can be integrated into the genome.

[24] Recovery of xanthan from a culture broth typically involves one or more processing steps. The xanthan may be heat-treated. The xanthan may be precipitated with an alchohol, such as isopropyl alcohol, ethyl alcohol, or propyl alcohol. Typically the cells are not specifically removed from the culture broth.

[25] Xanthan molecules produced biosynthetically typically have a distribution of sizes. The increased viscosity of the present invention may be achieved by increasing the number of molecules having a much longer than average length, or by increasing to a greater degree the number of molecules having a somewhat longer than average length. The number of molecules which have increased length need not be huge. At least 1, 3, 5, 7, 9, or 11 % of the molecules with an increased length may be sufficient. The molecules of increased length may be greater than 3, 4, 5, 6, 7, 8, or 9 um, as measured by atomic force microscopy. The percentage of the mass of the total xanthan population contributed by the molecules which are longer than 3, 4, 5, 6, 7, 8, or 9 um will be greater than their number proportion in the population. Thus at least 1, 3, 5, 10, 15, 20, or 25 % of the total mass of the xanthan molecules may be contributed by molecules having a greater than 3 um length.

[26] Intrinsic viscosity measurements are yet another way to characterize the preparations of the present invention. Increases seen using this type of measurement may be as great as 5, 10, 15, 20, 25, 30, or 35 % over that produced by wild-type strains. Proper controls for comparison purposes are those corresponding strains which are most closely related to the strains being tested. Thus if testing strains that have additional copies of *gumB* and *gumC,* the best control will have the same genetic complement but for the presence of the additional copies of *gumB* and *gumC.* If testing cultures that have been induced by an inducer to produce more *gumB* and *gumC* gene product, then the best control will be cultures of the same strain that have not been induced. Sea water viscosity can also be used to characterize preparations of the present invention. Increases seen using this type of measurement may be as great as 5, 10, 15, 20, 25, 30, or 35 % over that produced by wild-type strains.

[27] Xanthan is used as a component in a number of products to improve properties. The properties may include viscosity, suspension of particulates, mouth feel, bulk, to name just a few. Other properties include water-binding, thickener, emulsion stabilizing, foam enhancing, and sheer-thinning. Such products include foods, such as salad dressings, syrups, juice drinks, and frozen desserts. Such products also include printing dyes, oil drilling fluids,

ceramic glazes, and pharmaceutical compositions. In the latter case, xanthan can be used as a carrier or as a controlled release matrix. Other products where xanthan can be used include cleaning liquids, paint and ink, wallpaper adhesives, pesticides, toothpastes, and enzyme and cell immobilizers.

[28] While the invention has been described with respect to specific examples including presently preferred modes of carrying out the invention, those skilled in the art will appreciate that there are numerous variations and permutations of the above described systems and techniques that fall within the spirit and scope of the invention as set forth in the appended claims.

EXAMPLES

Example 1--Strain construction

[29] To isolate a fragment carrying the complete *gum* gene region of *X. campestris*, a genomic library of the wild type *X. campestris* strain, NRRL B-1459 (1), was constructed with the broad-host-range cosmid vector pRK311 (2) by cloning of total DNA partially digested with *Sau*3AI. This library was mated *en masse* from *E. coli S17-1* (3) to the Gum$^-$ *X. campestris* mutant 2895 (4). One of the cosmids isolated from several mucoid exconjugants termed pIZD15-261 (5) contains a 16-kb fragment encompassing the complete *gum* region. See Fig. 1 for a graphic representation and Table 1 for a listing of the genes of the operon.

Table 1

List of genes designations in the chromosomal region encoding xanthan polysaccharide synthesis

X. campestris ATCC13951 (NRRL B-1459)	*X.campestris pv. campestris* ATCC33913	Chromosomal Location*	Function
inf	himA (XCC2457)	2918744 - 2918448	integration host factor, alpha chain
orfX	(XCC2456)	2918464 - 2918111	transcriptional regulator
xpsB	gumB (XCC2454)	2917444 - 2916806	xanthan export
xpsC	gumC (XCC2453)	2916731 - 2915385	xanthan export
xpsD	gumD (XCC2452)	2915139 - 2913688	glucosyl transferase
xpsE	gumE (XCC2451)	2913602 - 2912307	xanthan polymerization
xpsF	gumF (XCC2450)	2912307 - 2911216	acetyl transferase
xpsG	gumG (XCC2449)	2911216 - 2910149	acetyl transferase
xpsH	gumH (XCC2448)	2910078 - 2908939	mannosyl transferase
xpsI	gumI (XCC2447)	2908939 - 2907893	mannosyl transferase
xpsJ	gumJ (XCC2446)	2907893 - 2906397	xanthan export
xpsK	gumK (XCC2445)	2906014 - 2905130	glucuronic transferase
xpsL	gumL (XCC2444)	2905086 - 2904295	pyruvyl transferase
xpsM	gumM (XCC2443)	2904284 - 2903496	glucosyl transferase
orf165	(XCC2442)	2903458 - 2902964	unknown conserved hypothetical

* Gene locations are according to the genome sequence of *X. campestris pv. campestris* ATCC33913 (GenBank deposition: AE008922) as described by da Silva, A. C. R., et al., (Nature, Vol. 417, pg. 459-463, 2002)

[30] For the construction of the pBBR5-BC plasmid, a 4026 bp fragment from pIZD15-261 digested with *Spe*I-*Bgl*II was cloned between the *Xba*I and *BamH*I sites of pKmob19 (8), giving rise to pGum02-19S (5). A 2855 bp fragment was released from plasmid pGum02-19S by digestion with *Sph*I. This fragment was cloned into pUC18 (9), which was previously digested with *Sph*I, forming pUC18-BCAS.

[31] The final plasmid (pBBR5-BC) was constructed by cloning the *Hin*dIII-*Xba*I fragment, containing the *gum* promoter and *gumB* and *gumC* genes, into *Hin*dIII-*Xba*I digested pBBR1-MCS5 (10) (GenBank accession no. U25061).

[32] The nucleotide sequence of the resulting pBBR5-BC plasmid is shown in SEQ ID NO: 1. (The predicted amino acid sequences of gumB and gumC are shown in SEQ ID NOs: 2 and 3, respectively. This broad-host-range, medium-copy-number plasmid is 7.6 kb in length and is compatible with IncP, IncQ and IncW group plasmids, as well as with Co1E1- and P15a-based replicons. The presence of an origin of transfer (*mobRK2*) enables its transference by conjugation into a wide range of bacteria when the RK2 transfer functions are provided in *trans*. It also carries the gentamicin resistance gene and it contains the pBluescript II KS multiple cloning site located within the gene encoding the LacZ α peptide (pBluescript II KS from Stratagene, La Jolla, Ca, USA).

[33] To verify the expression of GumB and GumC proteins from pBBR5-BC, the plasmid was introduced into *X. campestris* mutant 1231, in which the entire *gum (xps)* gene cluster was deleted. Both proteins were detected by Western blot in the mutant strain.

TABLE 2. Bacterial strains and plasmids used or constructed in this work.

Bacterial strain or plasmid	Relevant characteristics	Source (reference)
E. coli.		
DH5α	*F- endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 ΔlacU169 (φ80d lacZΔM15)*	New England Biolabs
S17-1	*E. coli* 294 RP4-2-Tc::Mu-Km::Tn7	(3)
JM109	*F' traD36 proA⁺B⁺ lacIᵠ Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14⁻ gyrA96 ompT hsdS$_B$(r_B^- m_B^-) gal [dcm] [lon]*	New England Biolabs
BL21(DE3)	*F- ompT hsdS$_B$(r_B^- m_B^-) gal [dcm] [lon] (DE3)*	Novagen
X. campestris		
NRRL B-1459	Wild type.	(1)
2895	Rif^r *xpsI-261*	(11)
1231	Tc::Tn 10 *ΔxpsI*	C.P. Kelco
XWCM1	Mutant of NRRL B-1459	C.P. Kelco
PRM-1	Mutant of NRRL B-1459	C.P. Kelco
Plasmids		
pRK311	*oriV*(RK2) Tc^r *oriT*(*mob*$^+$) *tra*$^-$ λ*cos lacZ*(α)	(2)
pIZD15-261	Cosmid based on pRK311 carrying the *X. campestris gum* region.	(5)
pK19mob	Km^r, pK19 derivative, *mob*-site	(8)
pgum02-19AS	pK19mob vector carrying the *gum* fragment 770-4795[a]	(5)
pUC18	Ap^r, ColE1, *lacZ*α$^+$	(9)
pUC18-BCAS	pUC18 vector carrying the *gum* fragment 770-3610[a]	This work
pBBR1-MCS5	Gm^r, pBBR1CM derivative, *mob*-site, *lacZ*α$^+$	(10)
pBBR5-BC	PBBR1-MCS5 carrying the *gum* fragment 770-3610[a]	This work
pQE-Xps#6	pQE30 vector carrying the *gum* fragment 1336-1971[a]	C.P. Kelco
pQE30	Ap^r	Qiagen
pREP4	Km^r	Qiagen
pET-C	pET22b(+) vector carrying the *gum* fragment 2135-3319[a]	This work
pET22b+	Ap^r	Novagen
pH336	pRK290 carrying gum BamHI fragments1-15052[a]	Synergen
pCOS6	pRK293 carrying SalI fragments 1-14585a and upstream xps I DNA	CP Kelco
pFD5	pRK404 carrying partial BamHI gum fragment 318-3464[a]	Ielpi
pCHC22	pRK293 carrying SalI fragments 1-9223a and upstream xps I DNA	(4)
pBBR-prom	pBBR1-MCS5 carrying gum fragment 1000-1276[a]	This work
pBBR5-B	pBBR1-MCS5 carrying gum fragment 770-1979[a]	This work
pBBR-promC	pBBR1-MCS5 carrying gum fragment 1979-3459[a]	This work

[a] Numbers correspond to the position in the nucleotide sequence of the *gum* region (GenBank, accession number U22511)

[34] Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 2. *E. coli* strains were grown in Luria-Bertani medium at 37° C. *X. campestris* strains were grown in TY (5 g of tryptone, 3 g of yeast extract, and 0.7 g of $CaCl_2$ per liter of H_2O) or in YM

medium (12) at 28°C. Antibiotics from Sigma (St. Louis, Mo.) were supplemented as required at the following concentrations (in micrograms per milliliter): for *X. campestris*, gentamicin, 30; and tetracycline, 10; for *E. coli*, gentamicin, 10; kanamycin, 30; ampicillin, 100; and tetracycline, 10.

[35] DNA biochemistry. Plasmid DNA from *E. coli* and *X. campestris* was prepared by using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). DNA restriction, agarose gel electrophoresis and cloning procedures were carried out in accordance with established protocols (13). All constructs were verified by DNA sequencing. Plasmid DNA was introduced into *E. coli* and *X. campestris* cells by electroporation as instructed by Bio-Rad (Richmond, Calif.) (used parameters: *E. coli*: 200Ω, 25μF, 2500V and *X. campestris*: 1000Ω, 25μF, 2500V).

[36] Analysis of nucleotide and protein sequences. The nucleotide and amino acid sequences were analyzed by using the MacVector Sequence Analysis Software (Oxford Molecular Limited, Cambridge, UK).

Example 2—Western analysis of *gumB* and *gumC* expression

[37] Western Analysis confirmed that gumB and gumC gene products are being over-expressed in the *X. campestris* strain with extra copies of *gumB* and *gumC*. See Figure 2.

Example 3—Intrinsic Viscosity determination

[38] Xanthan samples prepared from *X. campestris* strains with (XWCM1/pBBR5BC) and without (XWCM1) multiple, plasmid encoded copies of the *gumB* and *gumC* genes were compared. Shake flask fermentations, using glucose as a carbon source, were carried out to obtain xanthan from these strains.

[39] Intrinsic viscosity was determined by measuring viscosity on both purified and unpurified xanthan samples. An increase in the intrinsic viscosity for xanthan from *X. campestris* strain with multiple copies of *gumB* and *gumC* was observed. Intrinsic viscosity is proportional to the molecular weight for a given polymer type when measured under identical solvent and temperature conditions. Therefore, xanthan from *X. campestris* strain with multiple copies of *gumB* and *gumC* is of higher molecular weight compare to xanthan from control strain.

[40] Methods: Five shake flasks each of the two broths were tested. The broths of each type were combined and the total volume measured. The broth was then precipitated in isopropyl alcohol. (Note: It was estimated that the broth contained approximately 3% gum. Measuring the total broth volume and multiplying by 3% gave the approximate dry gum weight. This approximation was used to calculate the amount of water required to produce approximately a 0.5% gum solution). The wet fibers of the precipitate were then immediately rehydrated with mixing in 0.01M NaCl to produce approximately a 0.5% gum solution. The fibers were mixed for three hours with good shear using a 3-blade 2 inch diameter propeller stirrer, then allowed to stand overnight. The following procedure was used to prepare the samples for intrinsic viscosity measurements.

[41] Filter the ~0.5% gum solution, prepared above, using a Gelman Science 293mm pressure filtration unit. The solution is first filtered through a 20μ Magna nylon filter (N22SP29325) . The filter is pressurized to ~60 psi, and the solution collected into clean beakers. (Note: the filters are changed when the flow rate is reduced to ~ 5 drips per minute.

[42] Following the first filtration step, the samples are filtered two more times using the above filtration unit. First, through a Millipore 8.0μ filter (SCWP 293 25), then through a Gelman Versapor® 293 mm 1.2μ filter (66397). The filtered sample is recovered in clean beakers following each filtration step.

[43] After filtration, ~ 600 ml of the gum solution is placed into Spectra/Por® dialysis tubing 28.6 mm diameter Spectrum # S732706 (MWCO 12,000 to 14,000). The tubing is cut into lengths of ~ 18-20 inches, and a knot tied in one end. The solution is added to the tubing, filling it to within ~ 2 inches from the end. Tie a second knot in the tubing such that as little air as possible is trapped in the tubing. Continue until all the gum solution is in dialysis tubing.

[44] Rinse the outside of the tubing containing the gum solution for ~ 1 minute with de-ionized water, then place the tubing into a container of 0.01M NaCl. The salt solution should completely cover the dialysis tubing.

[45] Allow the tubing to sit in the 0.01M NaCl solution for 4 days, changing the NaCl solution daily. After the 4 days, cut open one end of the tubing and carefully transfer the gum solution to a clean beaker.

[46] Solids are run on the filtered dialyzed solution using the following procedure:

[47] Using an analytical balance capable of weighing to $\pm$ 0.0002 g, weigh and record the weight of a clean aluminum weighing dish VWR Cat #25433-008. (*A*)

[48] Using a clean pipet add approximately 10 ml of the gum solution to the aluminum pan and record the exact weight of the combined pan and gum solution. (*B*)

[49] Place the pan with the solution into a 105° C drying oven and allow to stand for 24 hours.

[50] Remove the pan from the oven after 24 hours, cool and reweigh. Record the weight of the pan and remaining dried gum. (*C*)

[51] Subtract the weight of the aluminum pan (*A*) from the weight of the pan plus the gum solution (*B*). Subtract the weight of the aluminum pan (*A*) from the weight of the dried gum plus the pan (*C*). Divide the first value (*B-A*) into the second (*C-A*). Multiply this value by 100 to obtain the % solids.

[52] Note: Solids were run in triplicate for each filtered dialyzed solution using the above procedure. The calculated % solids were than averaged for each sample and the averaged value was used.

[53] Based on the solids determination for each solution, the samples are diluted to 0.25% total gum concentration using 0.01M NaCl.

[54] Intrinsic viscosity measurements were made using the Vilastic Viscoelasticity Analyzer (Vilastic Scientific, Inc., Austin, TX, fitted with the 0.0537 cm radius X 6.137 cm length tube. The instrument was calibrated with water prior to making measurements and verified after the measurements were completed. Measurements were conducted using the instruments TIMET software protocol, set to a frequency of 2.0 Hz, a constant strain of 1.0, and an integration time of 10 seconds. The temperature was maintained at 23.5°C. The samples were prepared by dilution of the 0.25% gum solution. Each dilution was mixed for 20 minutes, and allowed to stand refrigerated overnight before being measured. Six measurements were made for each dilution and averaged. Table 3 below shows the dilutions and the resultant averaged viscosities for each prepared sample.

Table 3

	Dilutions		Viscosity Measurements	
Concentrations	0.25% X.G. (ml)	0.01M NaCl (ml)	XWCM1 Control	XWCM1/ pBBR5-BC
Solute 0.01M NaCl	0	100	.921	.921
0.0025%	1	99	1.114	1.165
0.0050%	2	98	1.326	1.486
0.0075%	3	97	1.537	1.829
0.0100%	4	96	1.762	2.181
0.0150%	6	94	2.302	2.963
0.0200%	8	92	2.920	3.901

[55] Intrinsic viscosities were determined by plotting the reduced specific viscosity (η_{sp}/c) against the gum concentration $\eta_{sp}/c = ((\eta_c - \eta_o) / \eta_o)$ where η_c = viscosity of the gum. The intercept yields the intrinsic viscosity. See Figure 3.

[56] The increase in intrinsic viscosity for the XWCM1/pBBR5-BC variant is believed due to an increase in molecular weight. Intrinsic viscosity is proportional to the molecular weight for a given polymer type when measured under identical solvent and temperature conditions as done in this experiment. The relationship between $[\eta]$ and molecular weight is given by the Mark-Houwink equation $[\eta] = kM^a$, where k and a are constants for a specified polymer type in a specified solvent at a specified temperature. Because the constant "a" is positive number, an increase in $[\eta]$ can only be obtained by an increase in the molecular weight (M) unless the samples have a different molecular conformation in which case the Mark-Houwink equation is not obeyed.

Example 4-- Procedure –Low Shear Rate Viscosity measurement

[57] Low shear rate viscosity measurements were performed on purified xanthan samples. The procedure used to measure LSRV is detailed below. Increased viscosity for xanthan from a strain with multiple copies of *gumB* and *gumC* compared to xanthan from a control strain was observed. The data suggest that over-expression of both *gumB* and *gumC* is required for increased chain length; over-expression of either gumB or gumC individually is not sufficient to increase chain length.

[58] Material and Equipment:

1. Standard (synthetic) Tap Water (water containing 1000 ppm NaCl and 40 ppm Ca^{++} or 147 ppm $CaCl_2.2H2O$): Prepare by dissolving in 20 Liters of distilled water contained in a suitable container, 20 gm of reagent grade NaCl and 2.94 gm of reagent grade $CaCl_2.2H_2O$.
2. Balance capable of accurately measuring to 0.01 gm.
3. Brookfield LV Viscometer, Spindle #1, and spindle Guard.
4. Standard laboratory glassware.
5. Standard laboratory stirring bench. An RAE stirring motor (C25U) and stirring shaft (5/16") with 3-bladed propeller may be substituted.

[59] Procedure:

1. To 299 ml of synthetic tap water weighed in a 600 ml Berzelius (tall form)
   beaker, slowly add 0.75 gm (weighed to the nearest 0.01 gm) of product,
   while stirring at 800 rpm.
2. After stirring four hours at 800 rpm, remove the solution from the stirring
   bench, and allow to stand for 30 minutes.

3. Adjust the temperature to room temperature and measure the viscosity using a Brookfield

LV Viscometer with the No. 1 spindle at 3 rpm. Record the viscosity after allowing the spindle to rotate for 3 minutes.

Example 5—Quantification of Protein Expression

[60] Cell lysates were subjected to Western blot and immunodetection analysis to establish the level of plasmid encoded GumB and GumC. Four independent blots were analyzed. Although absolute values for the same sample were not reproducible in each quantification, the relative quantities between samples remained the same in all the measurements.

[61] Preparation of antibodies raised against GumB and GumC. An 1184 bp DNA fragment encoding amino acid residues 53-447 of the GumC protein was produced by PCR amplification. The following primers were used: F2135: 5′GGAATTCCATATGTTGATGCCCGAGAAGTAC-3′ (SEQ ID NO: 4) and B3319: 5′CGGGATCCTCAAAAGATCAGGCCCAACGCGAGG-3 (SEQ ID NO: 5)′. The PCR product was digested with *Nde*I and *Bam*HI, subcloned into pET22b(+) and the resulting plasmid (pET-C) introduced into the *E. coli* strain BL21(DE3).

[62] *E. coli* BL21(pET-C) grown in L-broth containing 50 μg carbenicillin ml^{-1} to OD_{600} 0.6 was induced with 1 mM IPTG for 3h. Total cell lysates were prepared by treating with 1 mg lysozyme ml^{-1} in lysis buffer (50 mM Tris/HCl pH8, 1 mM EDTA pH8, 100 mM NaCl, 1 mM PMSF, 0.1 mg DNase ml^{-1}, 0.5 % Triton X-100) at 37° C for 30 min, followed by sonication on ice. Cell debris was removed by low speed centrifugation (Eppendof, 4000 x g, 5 min) and the supernatant was fractionated in a soluble and in a pellet (inclusion bodies) fraction by centrifugation at 14000 x g for 10 min. Pellet fraction was washed twice with lysis buffer, in a volume identical to that of the original cell lysate, once with 2 mg DOC ml^{-1} in lysis buffer followed by three washes with water. After treatment, proteins were separated by SDS-PAGE and the major band containing the overproduced GumC protein was cut and eluted for immunizing rabbits.

[63] *E. coli* JM109(pQE-Xps#6, pREP4) grown in L-broth containing 50 μg carbenicillin, 25 μg kanamycin ml^{-1} to OD_{600} 0.6 was induced with 1 mM IPTG for 3h. Total cell lysates were prepared by treating with 1 mg lysozyme ml^{-1} in lysis buffer (50 mM Tris/HCl pH8, 1 mM EDTA pH8, 100 mM NaCl, 1 mM PMSF, 0.1 mg DNase ml^{-1}, 0.5 % Triton X-100) at 37°C for 30 min, followed by sonication on ice. Cell debris was removed by low speed centrifugation (Eppendof, 4000 x g, 5 min) and the supernatant was fractionated in a soluble and in an pellet (inclusion bodies) fraction by centrifugation at 14000 x g for 10 min. Pellet fraction was washed twice with lysis buffer, resuspended in 6 M guanidine hydrochloride in 100 mM Phosphate buffer (pH7), 5 mM DTT, 5mM EDTA and inclusion bodies were chromatographed on an FPLC Superdex HR200 (Pharmacia Biotech) pre-equilibrated with buffer D (4 M GdnHCl, 50 mM Phosphate buffer (pH7), 150 mM NaCl). Fractions containing GumB were pooled and used to immunize mice.

[64] Construction of plasmids pFD5, pBBR-promC, and pBBR5-B. A 3141 bp fragment containing *gumB* and *gumC* genes was obtained by partial digestion of pIZD15-261 with *Bam*HI (#318 and #3459) and cloned into *Bam*HI-digested pRK404 to yield plasmid pFD5. A 1480 bp fragment was isolated by digestion of pGum02-19 with *Eco*RI (#1979) and *Bam*HI (#3459) and cloned in pBBR1MCS-5 previously digested with the same enzymes to yield pBBR-promC. Digestion of pGum02-19 with *Hin*dIII in the MCS and *Eco*RI (#1979) produced a 1233 bp fragment, which was cloned in pBBR1MCS-5 to yield plasmid pBBR5-B.

[65] New Zealand white female rabbits were immunized using GumC prepared as described above. A primary injection of 500 μg of the protein with complete Freund's adjuvant was given to the rabbits, followed by three injections of 250 μg of the protein with incomplete adjuvant on alternate weeks. BALB/c female mice were immunized using GumB prepared as described above. A primary injection of 100 μg of the protein with complete Freund's adjuvant was given to the mice, followed by three injections of 50 μg of the protein with incomplete adjuvant once a week. Polyclonal antibodies were prepared as

described by Harlow & Lane ((1999) *Using antibodies : a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and antisera were stored at -70°C. To obtain GumC-specific antibodies, the serum was adsorbed with both *E. coli* BL21(pET22b+) and Xc1231 acetone powders (Harlow & Lane, *supra*).

[66] Protein extracts. Plasmids were introduced into the parental strain PRM-1 by electroporation. The resulting strains were grown in YM medium at 28°C and 250 rpm to middle-logarithmic phase. Cells were harvested by centrifugation and the fresh-weight determined. The pellet was washed twice with 10 mM Tris/HCl, 10 mM EDTA (pH 8,0) to remove exopolysaccharide and resuspended in the same buffer at a concentration of 100mg/ml. After addition of 100µl Buffer A (10 mM Tris/HCl, 10 mM EDTA (pH 8.0), 1,5% SDS) to 50 µl of each sample, the mixture was incubated at room temperature for 10 min followed by incubation at 100°C for 12 min. Cell lysate was centrifuged at 14000 x g (Eppendorf 5415 C) for 5 min and the supernatant collected was designated as total protein extract. Protein concentration of each lysate was determined by the method of Markwell ((1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 87(1), 206-10) in the presence of SDS, using BSA as a standard.

[67] SDS-PAGE and inmunodetection. Cell lysates (30 µg per lane) were mixed with sample buffer (125 mM Tris/HCl, pH6.8; 4% SDS, 20 mM DTT, 0.05% bromophenol blue, 20% glycerol) and boiled for 2 min. Proteins were separated by SDS-10% polyacrylamide gel according to the method of Schägger and von Jagow ((1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* 166(2), 368-79). Electroblotting was performed using a semi-dry transfer system (Hoefer Semiphor unit) onto Immobilon-P membranes (PVDF, Millipore). The transfer was performed in a buffer containing 10 mM CAPS (pH11), 10% (v/v) methanol for 30 min at 2.5

mA/cm^2 of gel surface area. Once the electrotransfer was complete, the blots were stained with 0.5 % Ponceau-S red to assess the quality of the transfer and washed with Milli-Q®-grade water. The blots were blocked overnight at 4°C with 5% nonfat milk powder in TBST (150 mM NaCl, 10 mM Tris/HCl pH8, 0.05% Tween-20) (Harlow & Lane, *supra*) and then incubated with anti-GumB (1:3000) or anti-GumC (1:5000) antibodies in 3% nonfat milk powder in TBST at room temperature for 3h. Alkaline phosphatase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Sigma) were used for detection, respectively, as described by the manufacturer. The blots were washed three times with TBST and were developed in a solution containing nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP, Promega). Commercial protein markers MW-SDS-70L (Sigma) were used to calibrate SDS-PAGE.

[68] Blot quantification. The intensities of GumB and GumC protein bands were determined by scanning the NBT/BCIP developed filters with a UVP Densitometer (Ultra Violet Products) and quantified with GelWorks 1D Analysis software (NonLinear Dynamics Ltd). Each filter contained a reference lane of a PRM-1(pBBR-prom) extract to establish the level of chromosomally encoded GumB and GumC in the wild type cells. Relative amounts of GumB and GumC were observed. See Figs. 2A and 2B.

Example 6--Procedure – Molecular length or weight determination using Atomic Force Microscopy

[69] The direct visualization technique called Atomic force Microscopy (AFM) or Scanning Probe Microscope (SPM) was used to image the lengths of xanthan molecules from *X. campestris* strains with (XWCM1/pBBR5-BC) and without (XWCM1) multiple copies of *gumB* and *gumC*. The procedure used to

perform AFM is detailed below. We observed that the average molecular contour length of xanthan molecules produced by a strain with multiple copies of *gumB* and *gumC* was much longer than that of the parental strain.

[70] A 0.1 wt % of gum solution was prepared by mixing 0.1 g of gum in 100 gram distilled water for ~3 hours. A 1-ppm stock solution was prepared by diluting 20 µl of the 0.1 wt % solution into a 20g 0.1M ammonium acetate solution. 20 µl of the 1 ppm stock solution was sprayed onto freshly cleaved mica disc(s) (~1 cm^2). These mica sample disc(s) were then placed in a heated (~60 °C) vacuum chamber for ~ one hour to remove excess water. The dried mica disc(s) were then scanned using the Tapping Mode of the AFM. The molecular contour length of all AFM images was measured with the software provided by Digital Instruments.

[71] Contour lengths of population of xanthan molecules were measured. The results of this study are summarized in Table 4. (Molecules in each size class are less than or equal to the length indicated; the number of molecules indicated in a size class do not include the molecules counted in a smaller size class.) These results demonstrated that xanthan molecules from *X. campestris* strain with multiple copies of *gumB* and *gumC* were significantly larger then xanthan molecules from control strain. The atomic force microscopy (AFM) or scanning probe microscopy (SPM) was performed with a commercial instrument (Nanoscope IIIa, Digital Instruments, Santa Barbara, CA) using a silicon nitride cantilever tip.

Table 4

AFM Measurement of Xanthan Molecules
Contour Length

XWCM1				
Length* (μm)	Molecules (count)	Frequency (%)	Distribution	
			No. Avg.	Wt. Avg.
0.5	225	51.5	≤ 3 μm = 99.8%	≤ 3 μm = 98.7%
1	130	29.7		
1.5	40	9.2		
2	25	5.7		
2.5	13	3.0		
3	3	0.7		
3.5	0	0.0	> 3 μm = 0.2%	> 3 μm = 1.3%
4	0	0.0		
4.5	0	0.0		
5	1	0.2		
5.5	0	0.0		
6	0	0.0		
6.5	0	0.0		
7	0	0.0		
7.5	0	0.0		
8	0	0.0		
8.5	0	0.0		
9	0	0.0		
9.5	0	0.0		
10	0	0.0		
Total	437			

XWCM1/pBBR5-BC				
Length (μm)	Molecules (count)	Frequency (%)	Distribution	
			No. Avg.	Wt. Avg.
0.5	150	28.4	≤ 3 μm = 90.9%	≤ 3 μm = 70.9%
1	163	30.9		
1.5	82	15.5		
2	44	8.3		
2.5	29	5.5		
3	12	2.3		
3.5	12	2.3	> 3 μm = 9.1%	> 3 μm = 29.1%
4	13	2.5		
4.5	7	1.3		
5	4	0.8		
5.5	4	0.8		
6	0	0.0		
6.5	3	0.6		
7	2	0.4		
7.5	0	0.0		
8	0	0.0		
8.5	1	0.2		
9	1	0.2		
9.5	1	0.2		
10	0	0.0		
Total	528			

Example 7—Evaluation of seawater viscosity

[72] Xanthan produced by strain XWCM-1/pBBR5-BC was evaluated for seawater vi

[73] scosity (SWV), compared to a commercial xanthan product (Xanvis™). Typical SWV for Xanvis™ xanthan product is in the range of 18 to 22.

[74] Seawater viscosity was determined using the following procedure. Seawater solution was prepared by dissolving 41.95 g of sea salt (ASTM D1141-52, from Lake Products Co., Inc. Maryland Heights, Missouri) in 1 liter deionized water. 300 ml of seawater solution was transferred to a mixing cup that was attached to a Hamilton-Beach 936-2 mixer (Hamilton-Beach Div., Washington, D.C.). The mixer speed control was set to low and a single fluted disk attached to the mixing shaft. At the low speed setting, the mixer shaft rotates at approximately 4,000-6,000 rpm. 0.86 g of biogum product was slowly added over 15-30 seconds to the mixing cup and allowed to mix for 5 minutes. The mixer speed control was set to high (11,000 ± 1,000 rpm) and the test solution was allowed to mix for approximately 5 minutes. The mixture was allowed to mix for a total of 45 minutes, starting from time of biogum product addition. At the end of the 45 minutes mixing time, 2-3 drops of Bara Defoam (NL Baroid/NL industries, Inc., Houston, TX) was added and stirring was continued for an additional 30 seconds.

[75] The mixing cup was removed from the mixer and immersed in chilled water to lower the fluid's temperature to 25 ± 0.5°C. In order to insure a homogeneous solution, the solution was re-mixed after cooling for 5 seconds at 11,000 ± 1,000 rpm. The solution was transferred from the mixing cup to 400 ml Pyrex beaker and Fann viscosity (Fann Viscometer, Model 35A) was measured. This was accomplished by mixing at low speed (about 3 rpm). The reading was allowed to stabilize and then the shear stress value was read from dial and recorded as the SWV value at 3 rpm.

Table 5. Quality of XWCM-1/pBBR5-BC xanthan and Xanvis™ xanthan

Sample	*SWV* DR[a]
XWCM-1/pBBR5-BC	29 30
Xanvis™ xanthan	22

[a] dial reading

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